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Physical State and Expression of Human Papillomavirus in Laryngeal Carcinoma and Surrounding Normal Mucosa

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Epidemiologic and biomolecular evidence suggests that human papillomavirus (HPV) infection may be associated with the development of head and neck cancers. To clarify the role of HPV in larynx carcinoma, 25 patients were studied for the presence of viral DNA, possible virus integration into the cellular genome, and viral expression both in neoplastic tissues and in neighbouring normal mucosa. Twelve of 25 patients with neoplasia (48%) showed negative results for HPV sequences, and 13 (52%) showed positive results. Among the latter group of patients, seven were HPV-16 positive, five were HPV-6, and one was HPV-45. No multiple infections were detected. The physical status of the HPV genome was analysed by three methods: polymerase chain reaction (PCR), bidimensional agarose gel electrophoresis, and in situ hybridisation. Viral integration into the host genome occurred in 43% of cases of HPV-16 and in 20% of cases of HPV-6. Viral RNA expression was detected by reverse transcription–PCR only in HPV-16-positive tumours. The pattern of expression was consistent with an active role of HPV in cellular transformation. In conclusion, the present work suggests that HPV infection may be involved in some cases of laryngeal carcinoma. However, the transformation mechanisms might be different from those currently accepted for anogenital cancers. ***J. Med. Virol. 60:396–402, 2000.***

KEY WORDS: human papillomaviruses; larynx carcinoma; viral RNA expression; viral physical state

INTRODUCTION

Human papillomaviruses (HPVs) are known to cause cancer of the cervix and other anogenital sites. More than 75 different HPV genotypes have been cloned and characterised. The "low risk" HPVs (6, 11, 13, 32) are associated with benign lesions and have little potential for malignant progression. In contrast, high-risk HPV genotypes (16, 18, 31, 33, 35, 45) have marked malignant potential and are frequently associated with epithelial dysplasia and squamous cell carcinoma at several anatomic sites [Lorincz et al.,1992]. Genomic HPV DNA integration is usually present in malignant lesions of the cervix, resulting in the disruption of some portion of the E2 open reading frame (ORF) [Matsukura et al., 1989, Cullen et al., 1991; Park et al., 1997]. The product of the E2 ORF is a 48-kd protein with DNA-binding activity that plays an important role in regulating the expression of E6-E7 genes by suppressing the gene promoter p97/p105 of HPV-16/18 [Romanczuk et al., 1990]. The disruption of E2 ORF may cause increased transcription of E6 and E7 viral oncogenes. These two genes almost always are expressed in HPV-associated cancers of the cervix and interact with the tumour suppressor gene products p53 and pRB, altering the homeostasis of cellular growth control (Dyson et al., 1989; Scheffner et al., 1990). Recently, epidemiologic and molecular studies have suggested that HPV infection may also be associated with the head and neck cancer [for review see McKaig et al., 1998]. Despite the large number of reports about HPV prevalence in these anatomical regions, the role of HPV in the development of squamous cell carcinoma of the larynx is poorly understood. Moreover, it must still be proved that the molecular succession of events occurring in cervical carcinoma—virus integration, deletion of viral suppressor genes, and activation of viral oncogenes—takes place in larynx carcinoma. Twenty-five patients with larynx carcinoma attending the outpatient department of the Di-

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vision of Otolaryngology were studied for the presence of viral DNA, possible virus integration into the cellular genome, and viral expression both in neoplastic tissues and in neighbouring normal mucosa.

MATERIALS AND METHODS

Patients and Clinical Specimens

Larynx carcinoma biopsies were collected over a 2-year period from 25 consecutive patients (24 men and one woman; median age, 64 years; range, 38–74 years) attending the outpatient clinic of the Division of Otolaryngology. Each sample was divided into two: the first half was frozen at −80°C for DNA and RNA extraction, and the remainder was fixed in formalin for histologic examination. One verrucous and 24 squamous carcinomas were diagnosed. Staging was done according to TNM criteria: two patients had T1 N2 M0, six patients had T2 N0 M0, two patients had T2 N1 M0, three patients had T3 N0 M0, three patients had T3 N1 M0, three patients had T4 N0 M0, three patients had T4 N1 M0, and three patients had T4 N2 M0 disease. By histologic analysis on haematoxylin-eosin-stained sections, seven tumours were graded as well differentiated, seven as moderately differentiated, and nine as poorly differentiated/undifferentiated. Biopsy samples of neighbouring normal mucosa were available from 23 patients.

DNA and RNA Extraction

Total DNA and RNA were simultaneously extracted from samples by the Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH) commercial kit, according to the supplier's instructions. High yields of good-quality DNA were obtained from all biopsy samples, whereas nondegraded RNA was extracted from only 10 cases. This low percentage of available RNA may be related to the suboptimal conditions for rapid freezing in our outpatient clinic.

HPV Detection and Typing

HPV detection was achieved by polymerase chain reaction (PCR) with the consensus primers MY09 and MY11 (Perkin Elmer Italia, Italy), which amplify the conserved region of L1 ORF of a large number of HPVs [Manos et al., 1989]. Briefly, purified DNA was mixed with PCR buffer (50 mmol/L KCl, 2 mmol/L $MgCL_2$,10 mmol/L Tris-HCl at pH 8.3), 200 μmol/L dNTPs, and 500 nmol/L of each oligonucleotide primer. To avoid mispriming, the "hot start" strategy was adopted: after a 5-minute heat denaturation at 95°C, the temperature was lowered to 80°C, and 1.25 units of amplitaq DNA polymerase (Perkin Elmer Italia) was added for a final 50-μl volume. Samples were then subjected to 30 amplification cycles consisting of 60 sec at 94°C, 60 sec at 55°C, and 90 sec at 72°C in a 480 PCR-thermal cycler (Perkin Elmer Italia). PCR products were electrophoresed through 3% nu-sieve agarose gel (FMC BioProducts-Europe, Denmark) and visualised by ethidium bromide staining under ultraviolet light. Primers GH-20 and PC-04, amplifying a 260-bp fragment of the human β-globin gene, were used as a positive DNA control [Saiki et al., 1988]. In each amplification session, 50 ng of SiHa cell DNA was used as a positive control, and an equal amount of HaCaT cell DNA was used as a negative control.

For HPV typing, a second aliquot of DNA from the positive samples was amplified without the β-globin primers. The amplified products were assayed by restriction fragment length polymorphism (RLFP), according to Bernard et al. [1994]. The results of RFLP were confirmed by type-specific PCR designed to amplify the E6 region between nucleotide 118 and 493 for HPV-6 and between nucleotide 101 and 470 for HPV-16. Amplification conditions were those mentioned earlier, except that 35 amplification cycles were set. The amplified products were identified by hybridisation with a ^{32}P-labelled internal oligonucleotide probe.

Viral Genome Physical State

Three different methods were used. The first was a PCR assay with primers for the E2 region of the HPV genome [Das et al.,1992]. The amplification of a 1,134 bp indicates that the E2 region of HPV-16 is conserved and no genomic integration occurred, that is, the virus is in an episomal state. Conversely, the lack of amplified product indicates the absence of the target sequence and consequently the absence of episomal forms. The same strategy was used for HPV-6, using a set of primers amplifying a 1,027-bp fragment from the E2 region between nucleotide 2733 and nucleotide 3759. The reaction conditions were those used for consensus primer PCR.

The second method was bidimensional gel electrophoresis, as described elsewhere [Venuti and Marcante, 1989]. Total genomic DNA was digested with a noncutting restriction enzyme for the specific HPV type and sequentially electrophoresed through two agarose gels at different concentrations. Linear DNA (i.e., cellular DNA) yields a continuous crescent-shaped path, while the slowly migrating circular DNA (i.e., viral episomal DNA) produces a discrete spot out of the continuous track. The third method was in situ hybridisation with specific primers using the GenPoint kit (Dako, Carpinteria, CA) according to the manufacturer's instructions. Spotted or evenly distributed signals in the nuclei indicate the presence of integrated or episomal forms, respectively.

Expression of Viral Transcripts

Total RNA was subjected to reverse transcription (RT)-PCR using the Gene Amp RNA PCR kit (Perkin Elmer Italia). For HPV-16, primer sequences and PCR conditions were the same as those already published by Sherman et al. [1992]. To detect E6*I and E6*II RNAs, the primers indicated by Johnson et al. [1990] also were used. To detect HPV-6 mRNAs, primers for the region between nucleotide 118 and nucleotide 3738 were used for the first amplification and primers between nucleotide 139 and 3410 for the nested amplification. These primers allow for the detection of the

TABLE I. Larynx Carcinoma: Physical State of HPV-6 Sequence

Patient/site of biopsy	PCR Primers MY9/11	E6	E2	β-Globin	2D-EF	ISH
S./t	+	+	+	+	e	e
S/m++	+	+	+	+	ND	e
PA/t	+	+	+	+	e	e
PA/m++	+	+	+	+	ND	e
DDN/t	+	+	–	+	i	i
DDN/m	+	+	+	+	ND	e
DDN/m+	+	+	+	+	ND	ND
DDN/m++	+	+	+	+	ND	e
S.A./t	+	+	+	+	e	e
S.A./m	+	+	+	+	ND	e
S.A./m+	+	+	+	+	ND	ND
S.A./m++	+	+	+	+	ND	e
BC/t	+	+	+	+	e	e
BC/m	+	+	+	+	ND	ND
BC/m++	+	+	+	+	ND	e

PCR, polymerase chain reaction; t, tumour; m, tumour margin; m+, mucosa at 0.5 cm; m++, mucosa at 1 cm; ND, not done; 2D-EF, bidimensional electrophoresis; ISH, in situ hybridisation; e, episomal HPV DNA; i, integrated viral DNA.

TABLE II. Larynx Carcinoma: Physical State of HPV-16 Sequence

Patient/site of biopsy	PCR Primers MY9/11	E6	E2	β-Globin	2D-EF	ISH
DCA/t	+	+	+	+	e	e
DCA/m	–	–	–	+	ND	ND
DCA/m+	–	ND	ND	+	ND	ND
DCA/m++	–	ND	ND	+	ND	ND
SB/t	+	+	–	+	i	i
SB/m	+	+	–	+	ND	i
SB/m+	+	+	–	+	ND	i
SB/m++	+	+	+	+	e	e
CL/t	+	+	–	+	i	i
CL/m+	+	+	+	+	ND	e
CL/m++	–	ND	ND	+	ND	ND
DAG/t	+	+	+	+	e	e
DAG/m	+	+	+	+	ND	e
DAG/m+	+	+	+	+	ND	ND
DAG/m++	+	+	+	+	e	e
MORN/t	+	+	+	+	e	e
MORN/m++	+	+	+	+	ND	e
PC/t	+	+	–	+	i	i
PC/m++	+	+	+	+	e	e
ML/t	+	+	+	+	e	e
ML/m++	+	+	+	+	ND	e

PCR, polymerase chain reaction; t, tumour; m, tumour margin; m+, mucosa at 0.5 cm; m++, mucosa at 1 cm; ND, not done; 2D-EF, bidimensional electrophoresis; ISH, in situ hybridisation; e, episomal HPV DNA; i, integrated viral DNA.

most abundant mRNA species, named d, e, f, and m by to Chow et al. [1987]. PCR products of amplification were identified by size determination and by Southern blot analyses with internal oligonucleotide probe hybridisation. Samples were assayed twice in two independent sessions: specimens yielding two consecutive negative results were considered negative.

RESULTS

Presence and Typing of HPV DNA Sequences

HPV DNA sequences were detected in 13 of 25 larynx carcinomas (52%) by PCR with the consensus primers. Viral typing showed that HPV types 45, 16, and 6 were present in one, seven, and five samples, respectively (Tables I and II). The results of in situ hybridisation with specific probes confirmed these data. No multiple infection was detected, and no association between HPV type and differentiation grade of the tumour was found.

HPV Integration

Three methods were employed to determine the physical state of HPV DNA sequences in the tumour biopsies and in neighbouring normal tissues: PCR analysis to amplify the E2 region of the HPV genome (Fig. 1), bidimensional gel to ascertain the presence of slowly migrating circular DNA episomes (Fig. 2), and in situ hybridisation to show, through a diffuse or spotted nuclear stain, the presence of episomal or integrated HPV DNA, respectively. It was not possible in the case of all samples to use all three methods. HPV-16 genome was integrated in 42.8% (3/7) of samples, whereas the HPV-6 integration rate was 20% (Tables I and II). The only HPV-45 detected was integrated into the genome. In 23 cases we were able to collect and analyse samples from the surrounding mucosa. These samples were collected at the border of the neoplastic lesion (labelled "m"), at a distance of 0.5 cm (labelled "m+"), and at a distance of 1 cm (labelled "m++"). In no case were HPV DNA sequences detected in the normal mucosa of HPV-negative patients (data not shown). In 11 of 13 HPV-positive tumours, viral sequences were also detected outside the transformed area (Tables I and II).

In all cases, the same viral type was present in both normal and neoplastic tissue, indicating probable origin from a single infection. However, the particular anatomical site hampered the collection of samples from several distant areas, leaving unanswered the question of whether the patient suffered from a multifocal or diffuse infection. HPV DNA episomal forms were found in almost all normal tissue at the border and at 0.5 cm from the HPV-positive tumours, apart from patient DCA, who showed negative results for HPV DNA, and patient SB, who showed viral integration (Table II). Samples collected at 1 cm from the tumour showed differences from the closer normal mucosa in only two patients: in SB, the HPV-16 episomal form instead of the integrated form was present, and CL showed negative results for HPV-16 (Table II). Finally, HPV-6 was found to be integrated into the host genome in the tumour of patient DDN, with the normal mucosa showing the presence of episomal forms of HPV DNA (Table I).

HPV Expression

The presence of mRNA was preliminarily assayed by RT-PCR amplification of the GAPDH housekeeping gene mRNA. The presence of mRNA was detected in 10 samples: two with HPV-6, four with HPV-16, and four without HPV (data not shown). Specific viral tran-

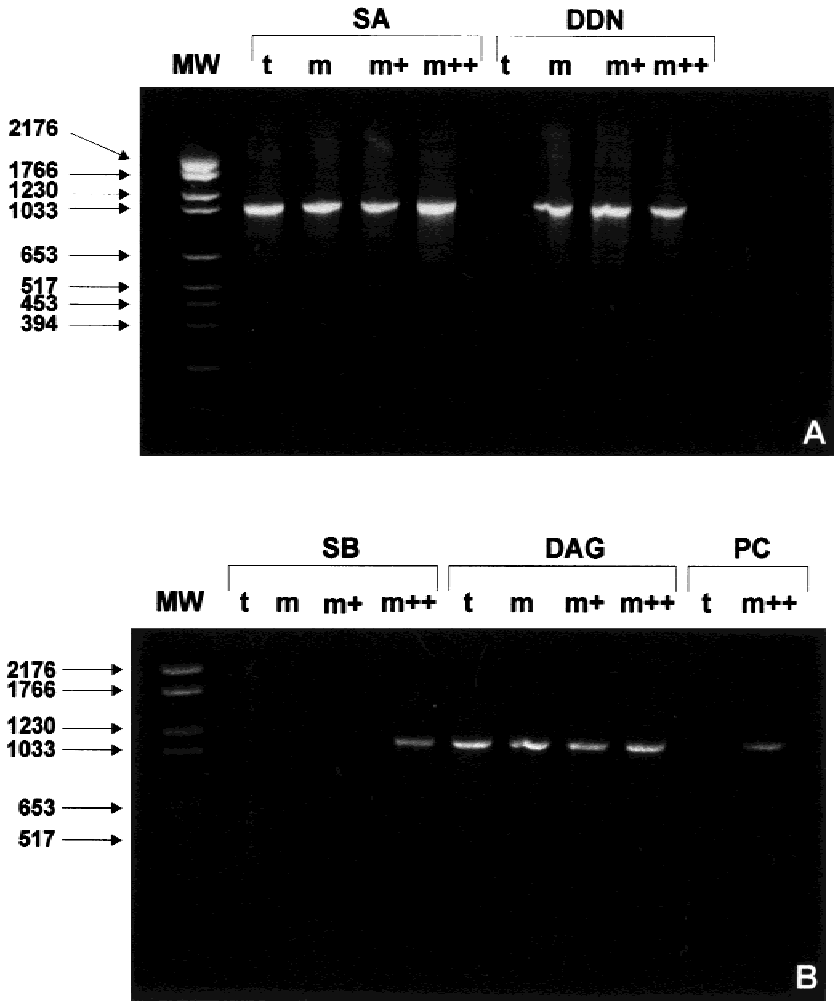

Fig. 1. Determination of HPV physical state by E2-targeted polymerase chain reaction according to Das et al. [1992]. The bands at 1,027 bp (**A**) and 1,139 bp (**B**) indicate the integrity of the E2 ORF of HPV-6 and HPV-16, respectively (i.e., episomal form). Letters across the top refer to the patient and the site of biopsy. t, tumour; m, tumour margin; m+, mucosa at 0.5 cm; m++, mucosa at 1 cm; MW, molecular weight markers. Base pair lengths are indicated on the left side.

scripts were then found in three of four cases of HPV-16 and in no case of HPV-6 (Table III). In patient DAG, carrying the virus in an episomal state both in tumour and in normal mucosa, the full-length E6 and the E6*I RNA species were present in tumour cells, whereas only the spliced forms E6*I, E6*II, and E6*III were expressed in normal mucosa. In patient DCA, in whom HPV DNA was present exclusively in tumour in episomal form, only E6*I was detected. Finally, the E6*III transcript was detected in patient PC both in tumour carrying the virus in integrated form and in normal mucosa, where the virus was episomal.

DISCUSSION

In a recent comprehensive review of head and neck squamous cell carcinoma, McKaig et al. [1998] reported that the overall prevalence of HPV positivity assessed by PCR was 34.5%. In the reviewed studies the number of tumours ranged from four to 187, and the HPV prevalence varied from 10% to 100%. In the present study, HPV DNA was detected in 52% of cases of laryngeal carcinoma in a population of 25 patients. The wide differences in primer choice, amplification conditions, hybridisation stringency, and other technical variables among reviewed studies account for such highly scattered results [Hoshikawa et al., 1990; Perez-Ayala et al., 1990; Clayman et al., 1994]. Nevertheless, several data indicate that the HPV prevalence rate does not differ in the larynx compared with other regions of the aerodigestive tract and that HPV may be involved in laryngeal carcinogenesis. In the anogenital region, a crucial event in HPV carcinogenesis is viral integration into the host genome. This event usually results in the disruption of some portion of the E2 ORF. The product of the E2 ORF is a 48-kd DNA-binding protein with regulatory and suppressive activity on the p97/p105 gene promoter of HPV-16/18 [Romanczuk et al., 1990]. Thus, E2 abrogation leads to E6 and E7 overexpression, an essential feature in the maintenance of the transformed state both in vivo and in vitro [zur Hausen, 1996]. Therefore, it seemed of interest to investigate whether this succession of events might also take place during carcinogenesis in the larynx. To this end, the type and the physical state of the viral genome were evaluated in samples of larynx carcinoma.

The results indicate that HPV integration into the cell genome appears to be a rare event, even in the late stage of carcinogenesis. Assuming that HPV has a role

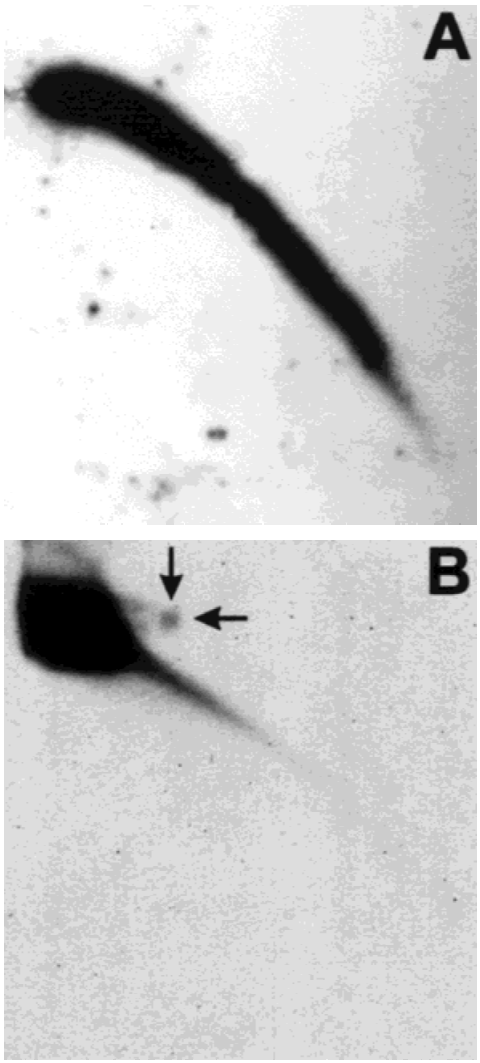

Fig. 2. Bidimensional agarose gel of DNA extracted from CL/t (**A**) and CL/m+ (**B**) samples (See Table II). The first run was in a 0.4% agarose gel and the second in a 0.8% agarose gel. Southern blot analysis was performed with a random primed ^{32}P-labelled probe for HPV-16. The arrows indicate the circular, episomal form of the viral DNA.

in larynx carcinogenesis, it is conceivable that despite the presence of conserved E2 sequences, E6/E7 overexpression is induced by the alteration of other, not yet identified genes involved in control of viral transcription and cell homeostasis. Considering the heavy exposure of the upper-aerodigestive tract to environmental carcinogens and the number of molecular targets potentially implicated, the search for such putative alterations is a fascinating but puzzling topic. In this regard, no association was found with tobacco smoking and/or alcohol drinking among our patients (data not shown), and there was no correlation between cancer development and p53 alteration, which seems to be an event that is independent of the presence of HPV [Badaracco et al., unpublished observations]. Although a high incidence of HPV nonintegrated forms has been reported for oral carcinoma, it has to be pointed out that bidimensional electrophoresis and E2-targeted

TABLE III. Larynx Carcinoma: HPV transcription

Patient/site of biopsy	HPV-16 mRNA				
	E6	E6*I	E6*II	E6*III	GAPDH
DAG/t	+	+	+	−	+
DAG/m	−	+	+	+	+
DAG/m+	−	+	+	+	+
PC/t	−	−	−	−	+
PC/m++	−	−	−	+	+
DCA/t	−	+	−	−	+
	HPV-6 mRNA				
	d[a]	e[a]	f[a]	m[a]	
DDN/t	−	−	−	−	+
DDN/m+	−	−	−	−	+
SA/t	−	−	−	−	+
SA/m++	−	−	−	−	+

t, tumour; m, tumour margin; m+, mucosa at 0.5 cm; m++, mucosa at 1 cm.
[a]mRNA species as described by Chow et al. [1987].

PCR are aimed at detecting episomal forms; therefore, the reported data cannot exclude the presence of integrated viral genomes. On the other hand, in situ hybridisation, which can show different patterns for integrated and episomal forms, is affected by low sensitivity. Thus, the present results are compatible with the anogenital model of viral transformation, considering that even a single integrated viral copy can provide a sufficient amount of deregulated E6/E7 transcript for full transformation [Smotkin and Wettstein, 1986].

A second remarkable point is the presence of a considerable number of carcinomas bearing "low risk" HPV-6 in episomal form. Two theories can be proposed to explain this finding. Either the HPV-6 detected in larynx carcinoma is a highly aggressive mutant or subtype with selective laryngeal tropism [Sang and Barbosa, 1992], or the high-risk and low-risk types have similar oncogenic potentials in the upper-respiratory tract that are still to be evaluated. Nonetheless, these findings are also compatible with the interpretation that HPV is only a passenger in the development of laryngeal carcinoma, representing a coincidental infection by a highly diffused epitheliotropic virus on an independently developed neoplastic lesion. To shed light on this topic, viral presence and physical status in tumours and in the surrounding normal mucosa were compared. On the basis of this analysis, at least four pathologic conditions were detected:

- Neoplastic lesions devoid of any HPV sequences within HPV-negative surrounding tissues
- Lesions in which tumour is HPV positive and normal mucosa is HPV negative
- Lesions in which both tumour and normal mucosa are HPV positive, but the virus is integrated only in the tumour
- Lesions in which tumour and normal mucosa are both HPV positive and contain the virus in the same physical status

In the second and third groups of lesions, the differences in the presence and physical state of HPV in

normal and neoplastic tissues may account for the involvement of HPV in tumour development. In the fourth group, however, viral sequences could be detected both in normal and neoplastic cells in the same physical status. It is possible that contamination occurred during sampling, though careful attention was paid and the scalpel blades were changed for each biopsy excision. Moreover, in situ hybridisation data confirmed the results of PCR assay. Assuming that HPV infection plays a role in this subset of tumours, a feature distinguishing normal from neoplastic tissues might be expected in the pattern of viral oncogene expression. To address this point, specific viral mRNA was assayed. As stated, specific viral mRNA was not detected in any HPV-6-positive cases, indicating that the viral sequences are not transcriptionally active in both episomal (patient SA) and integrated forms (patient DDN). On the contrary, viral transcripts were found in biopsy specimens from three of four HPV-16 cases available for analysis. This percentage, higher than that observed by other authors [Snjiders et al., 1997], may reflect the presence of a high viral burden in the collected tumours. In some HPV-16 RNA-positive samples, E6*I mRNA was detected. This transcript is easily detected in naturally occurring preneoplastic and neoplastic tissues [Sherman et al., 1992] and can direct the synthesis of the E7 protein in vivo [Doorbar et al., 1990]. This finding suggests that E7 protein plays a relevant role in larynx carcinogenesis. In this anatomical region, however, the presence of HPV seems to be less critical than in anogenital tissues, as underscored by the relevant fraction (48%) of HPV-negative tumours. E2 and the E1/E4 E5 transcripts—the latter the most abundant species in anogenital lesions—were not detected in any sample. This result indicates that the E1, E2 region is genetically silent and therefore has no control on E7 transcription, strengthening the hypothesis of the active role of this oncogene in larynx tumours.

The E6 co-linear RNA transcript was detected in only one tumour sample, whereas in three samples from adjacent normal mucosa the E6*III transcripts were found together with E6*I and E6*II mRNAs. In vitro translation of these spliced RNAs produces proteins that associate with p53 protein at a considerably lower affinity and are not able to promote its degradation [Shally et al., 1996]. Taken together, data on RNA transcription indicate that the pattern of E6 and E7 expression is compatible with the currently accepted model of anogenital viral carcinogenesis, at least in the examined HPV-16-positive larynx tumours.

In conclusion, results of the present work agree with data from the literature that underscores a relevant prevalence of HPV sequences in larynx carcinoma. The results with regard to the presence of high- and low-risk forms of HPV appear contradictory, however. The prevalence of low-risk HPVs and the lack of their transcription are not consistent with the current model of HPV carcinogenesis, suggesting that HPV represents a mere passenger in cancer development. On the other hand, the presence of active viral transcription in a number of cases of HPV-16 is in agreement with that expected according to the anogenital neoplasia model. Both questions remain open and might find answers in more extensive population-based studies.

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